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Screening of Potential Chemopreventive Agents Using Biochemical Markers of Carcinogenesis[1]

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ABSTRACT

Ninety potential chemopreventive agents were screened using 6 chemoprevention-associated biochemical end points. These compounds were tested using rodent (tracheal epithelial or liver) cells and human cells [neonatal foreskin fibroblasts, bronchial epithelial cells, or human leukemic cells (HL-60)]. The effects measured were: (*a*) inhibition of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced tyrosine kinase activity in HL-60 cells; (*b*) inhibition of TPA-induced ornithine decarboxylase (ODC) activity in rat tracheal epithelial cells; (*c*) inhibition of poly(ADP-ribose)polymerase in propane sultone-treated primary human fibroblasts; (*d*) inhibition of benzo[*a*]pyrene(B[*a*]P)-DNA binding in human bronchial epithelial cells; (*e*) induction of reduced glutathione in Buffalo rat liver cells; and (*f*) inhibition of TPA-induced free radical formation in primary human fibroblasts or HL-60 cells. Fifty compounds were highly effective in inhibiting TPA-induced tyrosine kinase activity. This assay identified compounds from a wide variety of chemical classes as effective inhibitors, including all the vitamins, retinoic acid analogues, protein kinase C inhibitors, and chemicals belonging to the amino acid category. Fifty-two chemicals were classified as highly positive compounds when examined for their ability to inhibit TPA-induced ODC activity. These agents showed a dose-dependent inhibition or inhibition at all doses. Retinoids, in general, exhibited strong inhibition of ODC activity. A category of compounds showing dose-dependent inhibition were the sulfur compounds, especially the thiols and thiones. Among the natural products, terpenes were strong inhibitors of ODC. Forty-seven compounds were classified as strong inhibitors of poly(ADP-ribose)polymerase. In the carcinogen-DNA binding inhibition assay, 21 compounds were identified as strong inhibitors, which include phenolic compounds as well as sulfur compounds. Vitamins and their analogues were also good inhibitors. Testing for induced glutathione yielded 19 compounds that were good inducers. Sulfur-containing compounds and most of the phenolic compounds were also inducers of glutathione. Twenty compounds were highly positive for inhibition of TPA-induced free radical formation. A significant number of phenolic and sulfur compounds were again strong oxygen radical scavengers. Some antiinflammatory agents were also identified as free radical inhibitors. In general, retinoids were quite active in all the assays. Eight compounds were positive in all of the six assays; these were vitamin C (ascorbic acid), bismuththiol, esculetin, etoperidone, folic acid, hydrocortisone, indole-3-carbinol, and tocopherol succinate. Agents that were positive in these assays may inhibit the carcinogenesis process by similar mechanisms in humans and are identified as candidates for development as chemopreventive agents. Agents capable of inhibiting multiple mechanisms are regarded as highly promising agents for cancer chemoprevention.

INTRODUCTION

One of the most promising areas in cancer research is chemoprevention. Chemoprevention is the process of inhibiting, delaying, or reversing the process of carcinogenesis and will ultimately provide enormous benefits to public health by lowering the incidence of human cancer. A large number of potential chemopreventive agents have been identified from epidemiological surveys, experimental preclinical and clinical observations, and structural homology with known chemopreventive agents. Multiple experimental preclinical systems are necessary to screen and analyze the efficacy of these agents before they can be evaluated in clinical trials. *In vitro* assay models using cells, subcellular factions, and tissues are being developed and validated for rapid initial screening. Biochemical markers of the carcinogenic processes are particularly useful in prescreening chemopreventive agents. In order to screen a large number of compounds for chemopreventive potential, a number of transformation-associated biochemical end points correlated with many stages in the carcinogenic processes were selected. These end points include interaction with the reactive metabolites of carcinogens, their metabolites or byproducts of the metabolic processes such as free radicals and oxidative processes, and alteration of specific enzyme expression or function. Not only are these biochemical marker assays useful in identifying chemopreventive agents but will provide insights into mechanisms of their action and will assist in the selection and prioritization of chemicals for additional development.

In this study, the results of screening 90 compounds for efficacy as chemopreventive agents are presented. The six biochemical assays used normal, primary, or immortalized rodent (tracheal epithelial and liver) cells and human cells (neonatal foreskin fibroblasts, bronchial epithelial cells, or human leukemic cells). The main prerequisite for the six biomarker assays was to use human cell lines whenever possible or cells which have a high level of a particular biomarker associated with any stage of carcinogenesis or which can be induced by a specific agent [a carcinogen (*e.g.*, propane sultone) or a tumor promoter (*e.g.*, TPA[3]). The rationale for this is that since the agents are screened for human chemopreventive potential, the cell culture systems used should be relevant to human situation. The following effects were measured: (*a*) inhibition of TPA-induced tyrosine kinase activity in human leukemic (HL-60) cells; (*b*) inhibition of TPA-induced ODC activity in rat tracheal epithelial (2C5) cells; (*c*) inhibition of PADPR in primary human fibroblasts; (*d*) inhibition of B(*a*)P-DNA binding in human bronchial epithelial (BEAS-2B) cells; (*e*) induction of reduced GSH in buffalo rat liver cells; and (*f*) inhibition of free radical formation in primary human fibroblasts or HL-60 cells.

In the first assay, tyrosine kinase inhibition was measured. It has been demonstrated that protein phosphorylation by tyrosine kinase is closely associated with cellular proliferation, and terminal differentiation is associated with a decline in tyrosine kinase levels (1). Tyrosine kinase activity also has been found to be associated with many growth factors such as epidermal growth factor, platelet-derived growth factor, and insulin, and with a number of onco-proteins including *src*, *erbB*, *fms*, *yes*, *neu*, *sis*, *abl*, *fes*, and *ras* (2, 3). In addition, strong correlation was observed between tyrosine kinase activity and the transforming ability of retroviruses. Therefore, it is quite possible that tyrosine kinase may play a role in transformation or

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[3] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ODC, ornithine decarboxylase; PADPR, poly (ADP-ribose)polymerase; B(*a*)P, benzo(*a*)pyrene; GSH, glutathione; TK, tyrosine kinase; DFMO, difluoromethyl ornithine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEG, polyethylene glycol.

in maintaining the transformed state of cells and that inhibition of tyrosine kinase may lead to suppression or reversal of carcinogenic processes. Human leukemic cells (HL-60 cells) were chosen for inhibition of TK activity as these cells have substantial levels of TK in their proliferative state (1) which can be further induced (3–4-fold) by TPA.

ODC inhibition was measured in a second assay. ODC is a rate-limiting enzyme in the synthesis of polyamines which appears to be a prerequisite for cell proliferation, differentiation, and neoplastic transformation. The induction of ODC has been suggested to play a significant role in tumor promotion. Initial studies with the mouse skin model (4, 5) and subsequently with other organ systems (6, 7) showed an excellent correlation between the induction of ODC activity and the tumor-promoting ability of a variety of substances. As tumor formation can be prevented by the agents that block induction of ODC, such as retinoids, DFMO, or inhibitors of arachidonic acid metabolism including indomethacin (8, 9), ODC inhibition was shown to be a promising tool for screening inhibitors of tumorigenesis (10). We have used 2C5 cells for ODC inhibition assay because these immortalized, nontumorigenic cells were derived originally from primary rat tracheal epithelial cells exposed to TPA (11) and could be induced to produce ODC with treatment of TPAV (12).

A third assay measured PADPR inhibition. PADPR is a nuclear enzyme that synthesizes protein-bound polymers of ADP-ribose using NAD as a substrate (13). Poly(ADP-ribosyl)ation has been suggested to be involved in DNA repair, sister chromatid exchanges, and cell differentiation (14). PADPR is rapidly modulated in response to mutagen treatment and probably represents one of the very early responses to DNA damage (15). Benzamide, a specific inhibitor of PADPR, prevented transformation in human fibroblasts in a cell cycle-specific manner (16) by physical agents and alkylating chemicals (17) at concentrations that inhibited PADPR synthesis but allowed no other side effects. Therefore, the ability to inhibit PADPR should be a pertinent property to help identify agents with chemopreventive activity. Primary human neonatal foreskin fibroblasts exposed to a direct carcinogen (propane sultone) showing 4–8-fold induction of PADPR activity when compared to the untreated control was selected to use in this study. As a positive inhibitor of PADPR activity, 3-amino benzamide (5 mM) showing 60–70% inhibition was used (preliminary studies).

Polycyclic aromatic hydrocarbons (PAHs) are a class of chemicals that contain many known carcinogens, including B(*a*)P. Certain occupations as well as tobacco smoke and charcoal-broiled foods contribute to the exposure of humans to these chemicals (18). A variety of mammalian cells have been shown to metabolize polycyclic aromatic hydrocarbons to polycyclic phenols, dihydrols, epoxides, quinones, and water-soluble conjugates by a series of carcinogen-metabolizing enzymes (19–21). These reactive intermediates are known to bind covalently to cellular macromolecules, including DNA and RNA in rodent and human cells, and the extent of binding seems to correlate with the carcinogenic potency of the hydrocarbons (22, 23). The presence of B(*a*)P-DNA adducts was reported in lung tissues from patients with lung cancer (24). Several plant phenols present in the human diet are known to inhibit carcinogenesis. Among them, ellagic acid was shown to inhibit binding of B(*a*)P to DNA, and its chemopreventive activity was explained as the process of forming adducts with B(*a*)P epoxide and thereby detoxifying it (25). Based on these findings, potential chemopreventive agents were screened for their ability to prevent the formation of DNA-carcinogen complexes, likely by inhibiting activating enzymes or by inducing detoxifying enzymes. A normal human bronchial epithelial cell line (BEAS-2B) that has been immortalized by infection with a hybrid virus preparation, adenovirus 12-SV40, was chosen for this study. Preliminary studies in our laboratory have indicated that BEAS-2B cells have carcinogen-metabolizing capabilities from their ability to activate [^{3}H]B(*a*)P and binding to DNA.

The induction of reduced GSH in rat liver cells was also used to screen potential chemopreventive agents. Glutathione has a variety of cellular functions, and one role of particular importance is the protection of cellular macromolecules against reactive intermediates (26). Because glutathione is a natural antioxidant, it can protect a cell during both initiation and promotion phases of carcinogenesis (27). Glutathione conjugation involves the reaction of electrophiles with the nucleophilic thiol of GSH, which decreases the availability of reactive electrophiles to bind to DNA and possibly initiate the transformation process. During the promotion phase, GSH can protect cells by limiting oxidative free radical attack. When large doses of GSH were given to the rats bearing aflatoxin-induced liver tumors, it resulted in substantial reduction of the tumors. Furthermore, butylated hydroxyanisole, a food additive, has been found to inhibit chemical carcinogenesis by increasing levels of GSH (28). These studies have provided strong evidence that GSH destroys free radicals leading to the reduction of reactive oxygen intermediates. Because GSH conjugation occurs mainly in the liver, a normal liver cell line [Buffalo rat liver cells (BRL 3A)] was chosen to screen agents in the GSH induction assay.

Reactive oxygen species such as superoxide radical anions, hydrogen peroxide, and hydroxyl radicals are produced by several biochemical reactions during the metabolism of molecular oxygen. It has been suggested that free radicals and related active species may play a role in tumor promotion (29). Free radicals and active states of oxygen are known to be capable of affecting genomes and are believed to be responsible for the carcinogenic effects of radiation and many chemical carcinogens (30, 31). Furthermore, it has been demonstrated that protease inhibitors that are known to inhibit tumor promotion are also capable of blocking free radical formation (32). Vitamin A derivatives have also been shown to inhibit tumor promotion, and similarly various retinoid compounds are active inhibitors of phagocyte O_2^- production (10). Therefore, it is quite possible that the administration of agents referred to as "free radical scavengers" may protect normal tissues from damage caused by free radicals. Primary neonatal human foreskin fibroblasts or HL-60 cells were chosen for free radical inhibition study because they were found to respond to TPA in generating O_2^- radicals in culture.

MATERIALS AND METHODS

Cells and Media

Human leukemic cells, BRL, and BEAS-2B cells were obtained from American Type Culture Collection. The 2C5 cells were derived from rat tracheal epithelial cells after treatment with TPA (11). F-12, minimal essential medium, Joklik's minimal essential medium, Waymouth's, RPMI, media supplements, and fetal bovine serum were purchased from GIBCO (Grand Island, NY).

Chemicals

Benzylisothiocyanate, bismuthithiol, phenethyl isothiocyanate, sodium selenate, sodium selenite, and silymarin were purchased from Aldrich Chemical Co. (Milwaukee, WI). The agent *N*-(4-hydroxyphenyl)retinamide was a gift from R. W. Johnson Pharmaceutical Research Institute (Springhouse, PA). Difluoromethylornithine was obtained from Marion-Merrell Dow Pharmaceuticals, Inc. (Cincinnati, OH), suramin from FBA pharmaceuticals (Westhaven, CT), ascorbyl palmitate from Alfa Products/Johnson Matthey (Ward Hill, MA), calcium glucarate from Fluka Chemical Corp. (Ronkonkoma, NY), chlorophyll from American Tokyo Kasei Inc. (Atlanta, GA), lovastatin from Merck Sharp & Dohme (West Point, PA), reduced GSH from Boehringer

Mannheim (Indianapolis, IN), and diallyl sulfide and diallyl disulfide were from Pfaltz & Bauer, Inc. (Waterbury, CT). Anethole trithione was a gift from Solvay Pharma (Suresnes, France). BASF 47851, carbenoxolone, dehydroepiandrosterone analogue 8354, etoperidone, oltipraz, RO16-9100, RO19-2968, β-sitosterol, temaroten, thiolutin, and α-tocopherol succinate PEG 1000 were obtained from the repository of the Division of Cancer Prevention and Control. The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): collagenase, ATP, TPA, *O*-phthaladehyde, histone, activated DNA, 3-aminobenzamide, NAD, superoxide dismutase, ferricytochrome c, polyglutamate:tyrosine (4:1), sodium orthovanadate, pyridoxal phosphate, dithiothreitol, ornithine, proteinase K, RNase T_1, RNase A, pepstatin, phenyl methyl sulfonyl fluoride, and the 63 remaining compounds tested for potential chemopreventive activity. [^{32}P]ATP, [^{3}H]B(*a*)P and [^{32}P]NAD were purchased from Amersham Corp. (Arlington Heights, IL) and [^{3}H]ornithine from New England Nuclear Research Products (Boston, MA).

Solubilization of Test Agents

The solubility of each test agent in medium was determined by adding fixed volumes of culture medium to a known amount of test agent. If the agent is insoluble in medium, then the agents are solubilized using appropriate nontoxic solvents before the addition to culture medium. For the majority of compounds that were not soluble in media or distilled water, dimethyl sulfoxide not exceeding 0.2% final concentration was used. In some cases, ethanol (*e.g.*, carbenoxolone, progesterone, retinol, and β-sitosterol) or tetra hydrofuran (*e.g.*, β-carotene and dimethyl prostaglandin E_2) not exceeding 0.2 and 0.1%, respectively, were also used (see Table 1 footnotes for solubility information). The test ranges included the highest soluble concentration in medium (up to 1 mM) or up to a nontoxic final concentration of 0.2% dimethyl sulfoxide, 0.2% ethanol, or 0.1% tetra hydrofuran.

Biochemical Assays

Assay for Tyrosine Kinase Inhibition. Human leukemic cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. The cells were grown in 500-ml roller bottles and allowed to reach a maximum density of 1×10^6 cells/ml. The cells were then plated at a density of 5×10^5 cells in 60-mm dishes and treated with TPA (0.1 μM in 0.001% dimethyl sulfoxide) alone for inducing TK activity or immediately followed by the addition of a positive control, biochanin A (10^{-3} M) or with five-log concentrations of the test agent (0.0001, 0.001, 0.01, 0.1, and 1 mM) for 24 h at 37°C with 5% CO_2.

Following incubation, the cells were collected by scraping, washed twice with phosphate-buffered saline, and resuspended at a density of 10^6 cells/ml in 5 mM HEPES buffer (pH 7.4). The cells were then resuspended in 1 ml of buffer containing 5 mM HEPES (pH 7.6), 1 mM $MgCl_2$, and 1 mM EDTA, and then placed on ice. The cell membrane was disrupted by sonication, and cellular debris was removed by centrifugation at 1000 × *g* for 10 min. The supernate was ultracentrifuged at 30,000 × *g* for 30 min at 4°C to recover the particulate fraction. The pellet was resuspended in 0.3 ml of buffer containing 25 mM HEPES, 5 mM 2-mercaptoethanol, and 0.1% Nonidet P-40. Samples were vortexed and centrifuged at 12,000 × *g* for 5 min, and the resulting supernatant (particulate fraction) was used for tyrosine kinase assay. Protein content was determined by the method of Lowry *et al.* (33) using bovine serum albumin as a standard.

Tyrosine phosphorylation was measured by a modification of the method developed by Frank and Sartorelli (1) using a synthetic polymer substrate, poly(Glu-Tyr) (4.1:0.9). Briefly, assay buffer of 100 μl contained 1 to 3 μg of protein, 20 mM HEPES (pH 7.6), 15 mM $MgCl_2$, 10 mM $ZnCl_2$, and 5% Nonidet P-40, 30 μM sodium orthovanadate, and with or without 40 μg poly(Glu-Tyr). After a 5-min incubation period at 25°C, the reaction was initiated by the addition of 15 μM [γ-^{32}P]ATP (3000 Ci/mmol). After 10 min, the reaction was stopped by the addition of 10 mM cold ATP. Fifty μl of the reaction mixture were spotted on 2.4-cm^2 glass microfiber filter discs and washed three times with cold 10% trichloroacetic acid containing 10 mM sodium pyrophosphate and one time with 95% ethanol, air dried, and counted in a scintillation counter. The net tyrosine kinase activity was determined after correcting for endogenous kinase activity.

Assay for Inhibition of Ornithine Decarboxylase. The 2C5 cells were maintained in Waymouth's media supplemented with 5% fetal bovine serum, 10^{-7} M hydrocortisone, and 10 μg/ml insulin. Cells were plated at a density of 1×10^6 cells/well in a 24-well tissue culture dish and allowed to incubate for 24 h at 37°C with 5% CO_2. Following the incubation, the media was removed and replaced with media containing TPA (0.5 μM) alone or TPA plus five-log concentrations up to 1 mM of the test agent or 10 μM DFMO as a positive inhibitor of ODC. The cells were incubated at 37°C for 5 h.

The medium was removed from the plates, and the cells were washed twice with cold phosphate-buffered saline. One hundred μl of 50 mM phosphate buffer containing 5 mM dithiothreitol, 0.1 M EDTA, and 40 μM pyridoxal phosphate were placed in each well, and the cells were subjected to three freeze-thaw cycles. Following the final cycle, the plates with the cell extracts were held on ice until the ODC assay was performed.

The ODC assay was based on the procedure of Djurhuus (34) by measuring the level of [^{3}H]putrescine synthesized from [^{3}H]ornithine. A reaction mixture of 190 μl containing 48 mM phosphate buffer (pH 7.2), 1 mM EDTA, 0.45 mM L-ornithine plus labeled tracer L-[2,3-^{3}H]ornithine (46.5 Ci/mmol), 0.25 mM dithiothreitol, 0.01 mM pyridoxal phosphate, and 50 μl of cell extract were incubated for 1 h at 37°C in 5% CO_2. The reaction was stopped by placing the samples on ice for 10 min. The samples were then transferred to Whatman p81 paper, a strong cation-exchanger, for selective binding of putrescine in excess ammonia (0.1 M) bath. After drying, the samples were counted in a liquid scintillation counter.

Assay for Inhibition of PADPR. Human foreskin tissues were supplied by Rex Hospital (Raleigh, NC). Primary human fibroblast cultures were prepared as follows. The tissues were minced after rinsing in Joklik's minimal essential medium supplemented with fungizone (2 μg/ml) and gentamicin (25 μg/ml) and incubated with 0.5% collagenase for 45 min at 37°C. The cells were washed two times in minimal essential medium supplemented with serine (21 μg/ml), aspartic acid (13.3 μg/ml), and sodium pyruvate (110 μg/ml) and plated in 75-cm^2 tissue culture flasks with the above minimal essential medium supplemented with 10% fetal bovine serum and incubated at 37°C in 5% CO_2. The cells were subcultured upon confluency and used between the third and sixth passage.

Cells (1×10^6) were treated with propane sultone (41 nM) alone or in the presence of a positive inhibitor, 3-aminobenzamide (5 mM), or five-log doses of a test agent at the same time for 18 h at 37°C in 5% CO_2. The medium was removed, and the cells were washed two times with basic buffer (0.6 M NaCl, 50 mM Trizma base, 1 mM EDTA, 0.5 mM dithiothreitol, and 10 mM sodium bisulfite). The cells were scraped into basic buffer containing 0.1 M phenylmethyl sulfonyl fluoride and 1 mM pepstatin and sonicated twice for 5 s in ice and centrifuged at 10,000 × *g* for 10 min. An aliquot was removed for protein determination by the Lowry method (33), and the samples were held on ice until assayed.

The assay used for PADPR determination was a modification of the procedure developed by Scovassi *et al.* (35). A reaction mixture for each sample was prepared in a total volume of 100 μl containing the following: 50 mM Trizma base (pH 8.0), 10 mM magnesium acetate, 1 mM dithiothreitol, 2 μg activated DNA, 2 μg H1 histone, 1 mM NAD, 1 μCi [adenine-2,8-^{32}P]NAD (1000 Ci/mmol), and 30 μg of extracted protein. Following a 10-min incubation at room temperature, the samples were spotted on 2.4-cm glass microfiber filters treated previously with 1% bovine serum albumin. The samples were washed three times with 5% trichloroacetic acid containing 2% pyrophosphate and one time with 95% ethanol. The filters were air dried and counted in a liquid scintillation counter.

Inhibition of Carcinogen-DNA Binding Assay. BEAS-2B cells were maintained in 75-cm^2 tissue culture flasks with LHC-8 media supplemented with retinoic acid (0.1 μg/ml) and epinephrine (0.5 μg/ml). The cells were passaged when they reached approximately 80% confluence and were maintained at 37°C in 5% CO_2.

BEAS-2B cells were plated at a density of 5×10^5 cells/well in 6-well tissue culture plates and allowed to incubate for 18 h at 37°C in 5% CO_2. Cells were treated with 1 μM [^{3}H]B(*a*)P alone for 4 h at 37°C or after pretreating with five-log dilutions of a test agent or with ellagic acid (0.1 mM) for 2 h. Following incubation, the DNA was harvested by the method described by Davis *et al.* (36). Briefly, the cells were washed twice in proteinase K (100 μg/ml) in Tris-EDTA buffer containing 0.2 M Trizma, and 0.1 M EDTA (pH 8.5) was added to each well; the cells were allowed to detach for 10 min at 37°C. After the incubation, the cells were transferred to 25 μl of 20% sodium dodecyl sulfate and incubated for 3 h at 55°C and placed on ice; 75 μl of 5 M potassium acetate was added to each tube. Following a 30-min incubation on

ice, the tubes were centrifuged for 15 min at 15,000 × *g*. To the supernate, 2.5 ml of ice-cold ethanol was added to precipitate the DNA overnight at −20°C, and the DNA was resuspended in Tris-EDTA buffer. RNA was removed by treating with RNase T1 and RNase A at 37°C for 1 h. An aliquot was used for determining the DNA content by the absorbance at 260 nm, and the rest of the sample was used to determine the radioactivity; the percentage inhibition of carcinogen-DNA binding was determined by measuring the cpm/mg of DNA in the carcinogen-treated *versus* untreated samples.

GSH Assay. Buffalo rat liver (BRL3A) cells were maintained in F-12 media supplemented with 5% fetal bovine serum. Cells were seeded at a density of 1×10^6 cells per 60-mm dish in F-12 media supplemented with 1% fetal bovine serum for 24 h, and five-log concentrations of the test agents up to 1 mM were added to the cells for induction of GSH. The plates were then incubated for an additional 24 h.

The plates were washed two times with cold phosphate-buffered saline; following the washes, 1 ml of 0.25 M sucrose buffer containing 5% trichloroacetic acid was added to each plate. The cells were scraped, transferred to microcentrifuge tubes, and sonicated two times at a setting of 35 for 10 s to disrupt the cell membrane. The samples were then ultracentrifuged at 100,000 × *g* at 4°C for 30 min. The supernate was removed and stored at −70°C until assayed for GSH.

GSH levels were measured by a method developed by Hissin and Hilf (37) where GSH in the sample was reacted with *O*-phthaladehyde to form a fluorescent product that is activated at 350 nm with an emission peak at 420 nm. Briefly, 1.8 ml of phosphate (0.1 m) EDTA (5 mM) buffer was added to each tube. Cell supernate (100 μl) was then added to the buffer, followed by 100 μl of *O*-phthaladehyde (1 mg/ml in methanol), and allowed to react for 15 min at room temperature. The fluorescent product was then measured at 420 nm.

Free Radical Inhibition. A modification of the method developed by Pick and Mizel (38) was used to detect the inhibition of free radical formation. Cells (1×10^6 HL-60 cells or 5×10^4 primary human fibroblasts) were plated in 96-well tissue culture dishes suspended in Hanks' balanced salt solution. When primary human fibroblasts were used, they were allowed to attach for 30 min. Free radical formation was induced by the addition of TPA (8 μM), followed by an addition of five-log concentrations of chemopreventive agents up to 1 mM at the same time as well as cytochrome c (160 μM), and incubated at 37°C for 20 min. Bovine serum albumin was used as a blank, and superoxide dismutase (700 units) was used as a positive inhibitor. Cytochrome c reduction was measured at 550 nm using 620 nm as a reference. Using induction by TPA alone as a measure of maximum free radical formation, the percentage of radical formation in TPA plus agent-treated samples was determined.

RESULTS

A summary of screening results for 90 compounds in six biochemical assays is presented in Table 1. The effects of compounds in each assay were classified into four categories, based on the degree of inhibition, and denoted as −, +, ++, and +++, indicating no effect to strongest inhibition or induction.

Inhibition of Tyrosine Kinase. Of the 90 compounds assayed, 50 were highly inhibitory and given a three plus (+++) score. All compounds in this category (+++) showed one or more of the following features: (*a*) a dose-dependent inhibition; (*b*) at least 20% inhibition at all doses; (*c*) 100% inhibition at two doses; and (*d*) more than 50% inhibition at three doses. No one particular chemical class was active, but many of the vitamins (A, B, C, D, and K) were highly active. Vitamin E was marginally effective. Thirteen compounds showed greater than or equal to 20% inhibition in two doses and were given a two plus (++) score. Twenty-five compounds showed marginal inhibition of TPA-induced enzyme activity by having only one dose inhibitory and were given a one plus (+) score. Thirteen compounds were classified as negative (−) because they showed less than a 20% inhibition of TPA-induced enzyme activity.

Inhibition of Ornithine Decarboxylase. Fifty-two chemicals were classified as highly positive compounds (+++) because they showed a dose-dependent inhibition or inhibition at all doses. A wide variety of chemical classes including DFMO were highly active. Compounds that inhibited 60 to 100% at one or more doses were also included in the same category. Seventeen compounds showing an inhibition of more than 30 to 60% were included in the "++" category. Eight chemicals that showed 15 to 30% inhibition of TPA-induced enzyme activity at one or more doses were rated as "+." Thirteen compounds indicated as "−" showed either negative or less than 15% inhibition at all the doses tested.

Inhibition of PADPR. If all of the doses showed above 20% inhibition or three doses above 60% inhibition, these compounds were classified as strong inhibitors (+++); and 47 compounds belonged to this category. Twenty-one compounds elicited a 40 to 60% inhibition in two or more doses and were rated as "++." Twelve compounds, which showed a 20 to 40% inhibition of propane-induced PADPR activity in one or two doses, were put under the category of "+." Eleven compounds were either negative or exhibited less than 20% inhibition (−).

Inhibition of Carcinogen-DNA Binding. Twenty-one compounds were rated as strong inhibitors (+++), where either three doses showed inhibition above 20% or two doses above 40%. Vitamins A and E were highly active. Nine compounds showed above 25% inhibition (++) in either one or two doses. Fifteen compounds showing more than 15% inhibition in a single dose were rated as "+." Forty-five compounds were either negative or showed less than 15% inhibition of B(*a*)P-DNA binding.

Induction of Reduced GSH. The level of induced GSH was measured in Buffalo rat liver cells, where the basal level of GSH without a test agent was taken as 100%. A compound is considered as a positive inducer only if the induction is more than 10% of the level in cells alone. Nineteen compounds were rated as high inducers (+++) based on their induction at all doses or induction by three doses of more than 30%. Sulfur-containing compounds such as *N*-acetyl-L-cysteine and diallyl sulfide were highly active. Two doses above 20% or one dose above 30% induction were elicited by 21 compounds and denoted as "++." One dose of nine compounds showed more than 10% of the control cells and were rated as "+." Thirty-eight compounds were negative (−) in the assay, meaning that the induction level was equal to or less than 10% above that of the control cells.

Inhibition of Free Radical Formation. Inhibition of TPA-induced, free radical formation was measured in primary human fibroblasts or HL-60 cells. A compound was regarded as positive if it inhibited more than 10% of the promoter-induced free radical formation in one or more doses. Only 20 compounds were considered highly positive (+++) because either all doses were inhibitory or two doses showed more than 30% inhibition. Twenty-two compounds that showed inhibition in two or more doses were ranked as "++." Nine compounds showed more than 10% inhibition, and were rated as "+." Again, a wide variety of compounds, including vitamin E and β-carotene, highly reduced free radical formation. Thirty-nine compounds showed no inhibition in any of the doses or showed inhibition of less than 10% of TPA-induced free radicals. Among the compounds tested, eight were positive in all of the six assays: ascorbic acid (vitamin C), bismuthhiol, esculetin, etoperidone, folic acid (vitamin B_c), hydrocortisone, indol-3-carbinol, and tocopherol succinate (Table 2).

DISCUSSION

This study was designed to provide a large amount of mechanistic data to rapidly screen a large number of compounds for chemopreventive potential using six carcinogenesis-associated biochemical end points. Inhibition of TPA-induced tyrosine kinase activity was studied

Table 1 *Assay response of in vitro biomarker assays summary results*

Compound	TK[a]	ODC[b]	GSH[c]	FR[d]	PADPR[e]	DNA binding[f]
N-acetyl-L-cysteine[g]	+++	++	+++	+	++	–
Anethole trithione[h]	–	+	++	–	+++	–
Ascorbyl palmitate[h]	+++	+++	+	+	++	–
Aspirin[g]	+	+++	–	++	+++	+++
BASF 47851[h]	++	+++	–	–	–	+++
Benzyl isothiocyanate[h]	++	+	+	–	–	+++
Bismuththiol I[h]	+++	+++	+	+++	+++	+++
Butyrate, sodium[g]	+++	+++	–	+++	++	+
Caffeic acid[g]	–	+++	+++	++	+	++
Calcium-D-glucarate[h]	+++	+++	–	++	+++	++
Carbenoxolone[i]	–	+++	–	–	+++	+++
L-Carnosine[g]	+	+++	+++	–	+++	+
β-Carotene, *trans*[j]	+++	+++	–	+++	+++	+++
Catechin[h]	+++	+	++	–	+++	+++
Chlorogenic acid[g]	–	++	+++	–	+	–
Chlorophyll[g]	+++	–	–	–	–	–
Curcumin[h]	–	–	+++	–	+++	+
Difluoromethyl ornithine[g]	+++	+++	++	–	+++	++
Dehydroepiandrosterone analogue 8354[h]	–	+++	–	++	–	–
Dehydroepiandrosterone[h]	–	–	+++	+++	+	–
Diallyl disulfide[h]	++	–	++	++	++	–
Diallyl sulfide[h]	+	+++	+++	++	–	–
Dimethyl prostaglandin E_2[j]	–	+	++	++	+++	+++
Ellagic acid[g]	++	–	++	–	++	+++
Esculetin[h]	+	+++	+	+	++	+++
Ethyl vanillin[g]	+++	–	–	–	++	+++
Etoperidone[h]	+++	+++	+	++	+++	+++
Fluocinolone acetonide[g]	+++	–	–	–	++	+++
Folic acid[g]	+++	+++	++	++	+++	++
Fumaric acid[g]	+++	+++	–	–	+++	–
Glucaric acid[g]	+++	+++	+	+++	+++	–
Glucaro-1,4-lactone[g]	+++	–	++	+	+++	–
18-β-Glycyrrhetinic acid[h]	++	++	–	+++	+++	+
N-(4-Hydroxyphenyl) retinamide[h]	+++	+++	+	+++	+++	–
Hydrocortisone[h]	+++	+++	+	+++	+++	+
Ibuprofen[g]	+++	–	–	–	++	++
Indole-3-carbinol[i]	+++	+	++	+++	+++	+++
Indomethacin[h]	+++	+	+++	–	++	+
Levamisole[g]	–		–	–	+++	–
D-Limonene[h]	++	++	–	++	+++	–
Lovastatin[h]	++	+	–	+++	+	+
2-Mercaptoethane sulfonic acid[g]	–	+++	+	++	+++	++
Miconazole[h]	–	+++	–	++	++	++
Molybdate, sodium[g]	++	+++	–	+++	+++	+
Nicotinic acid (Vit. B_3)[g]	+++	+++	+++	++	+	–
Nordihydroguaiaretic acid[h]	+++	+++	++	+	–	–
Oltipraz[h]	+++	–	+++	+	–	++
2-Oxothiazolidine-4-carboxylate[g]	+	+++	+	+++	+++	–
Palmitoylcarnitine HCl[g]	+++	+++	++	–	+++	–
Phenethyl isothiocyanate[h]	–	++	–	–	+	–
Phenidone[h]	+++	++	–	–	+++	+
Piroxicam[h]	+++	++	–	–	+++	+
Potassium glucarate[g]	+++	+++	–	–	+++	–
Praziquantel[h]	+++	+++	–	–	+++	–
Prednisone[h]	+++	++	–	+++	++	–
Progesterone	+	+++	+++	++	++	–
Promethazine[h]	+	+++	++	–	+++	+
Propyl gallate[h]	++	++	++	+++	++	–
Purpurin[h]	+++	+++	–	+++	++	–
Quercetin[h]	+++	+	–	–	++	–
Retinoic acid, *all trans*[h]	+++	++	–	++	++	+++
Retinol	+++	+++	++	+++	+++	–
Rhodamine B[g]	–	+++	–	–	+++	–
Riboflavin-5-phosphate[c]	–	++	+	–	+++	–
RO 16-9100[h]	+++	+++	–	++	+	+++
RO 19-2968[h]	+++	+++	–	++	+++	+
Rutin[h]	++	+++	–	–	+++	–
Selenate, sodium[c]	+++	+++	–	++	+++	–
Selenite, sodium[g]	++	+++	+++	++	–	+
DL-Selenomethionine[g]	+++	++	–	–	++	–
L-Selenomethionine[c]	++	+++	+	–	+++	–
Silymarin[h]	+	+	–	–	–	–
β-Sitosterol	+++	+++	–	–	+++	–
Suramin, sodium[g]	+++	++	++	–	+	+++
Tamoxifen[i]	+++	+++	+++	–	+++	–
Taurine[g]	+++	++	++	–	+++	–
Temaroten[h]	+++	++	+++	+++	–	–
Transforming growth factor-β[g]	+	+++	+++	+	+	–
Thioctic acid	+++	–	++	++	+	–
Thiolutin[h]	+	+++	++	+++	–	+++
Thiosulfate, sodium[g]	+++	+++	+++	–	+++	–
α-Tocopherol acetate[h]	+	+++	–	+++	+	+

Table 1 *Continued*

α-Tocopherol succinate PEG 1000[h]	++	+++	–	+++	+++	+++
α-Tocopherol succinate[h]	+	++	++	++	+++	+++
Vanillin[g]	+++	++	+++	–	++	++
Verapamil[h]	+	++	+++	–	++	–
Vitamin C[g]	+	+++	++	+	++	+
Vitamin K_3[h]	+++	+++	–	+	+++	++
Vitamin D_3[h]	+++	+++	+++	–	+++	–
(*N*-6-Aminohexyl)-5-chloro-1-napthalenesulfonamide[h]	+++	+++	–	++	+++	–

[a] Inhibition of TK: +++, dose-dependent inhibition or all doses inhibitory or two doses show 100% inhibition or three doses show greater than 50% inhibition; ++, two or more doses inhibitory; +, one dose only inhibitory and showing 20% or more inhibition of TPA-induced activity; [minus], no inhibition or inhibition less than 20% of TPA-induced enzyme activity.

[b] Inhibition of ODC: +++, dose-dependent inhibition or all doses inhibitory or two doses show inhibition between 60% and 100%; ++, two or more doses show between 30% and 60% inhibition; +, one or more doses show between 15 and 30% inhibition; [minus], all doses show less than 15% inhibition.

[c] Induction of reduced glutathione: +++, all doses induce or one dose induces above 30% of media control or three doses induce 30% above media control; ++, two doses induce 20% above media control; +, one dose induces at least 10% above media control; [minus], no induction.

[d] Free radical inhibition: +++, all doses inhibitory or two or more doses show greater than 30% inhibition; ++, two or more doses inhibitory; +, one dose inhibitory ([mt]10%); [minus], no inhibition or less than 10% of TPA-induced free radical formation.

[e] Inhibition of PADPR: +++, all doses inhibitory or three doses show between 60 and 100% inhibition; ++, two or more doses show between 40 and 60% inhibition; +, one or more doses show between 20 and 40% inhibition; [minus], no inhibition or inhibition less than 20% of propane sultone-induced activity.

[f] Inhibition of carcinogen-DNA binding: +++, all doses inhibitory or one dose above 25% or three doses show greater than 20% inhibition or two doses show greater than 40% inhibition; ++, two doses inhibitory; +, one dose inhibitory and inhibition greater than 15%; [minus], no inhibition or inhibition less than 15% of B(*a*) P-DNA binding.

[g] Compound dissolved in media.

[h] Compound dissolved in DMSO.

[i] Compound dissolved in ethanol.

[j] Compound dissolved in tetrahydrofuran.

using HL-60 cells. The cell line was reported to be responsive to TPA in inducing tyrosine kinase activity. Extensive research efforts were needed to standardize and modify the assay procedures to make them reliable and sensitive for screening agents with chemopreventive potential. Modifications of the protocol by Frank and Sartorelli (1) included reduction of the TPA exposure time from 48 to 24 h, which resulted in an 11-fold increase in tyrosine kinase activity as well as a decrease in the toxicity of a number of chemicals.[5] At 10^{-7} M TPA, a 3-fold induction of tyrosine kinase activity was observed. This assay detected a large number (50 of 90) of compounds as highly positive inhibitors. Compounds were identified from a wide variety of chemical classes. Many vitamins, including retinoic acid analogues, were positive in the assay (*e.g.*, folic acid, 4-hydroxyphenyl retinamide, vitamin C, vitamin K_3, vitamin D_3, β-carotene, *trans*-retinoic acid, retinol, RO16–9100, RO19–2968, and temaroten). A number of known protein kinase C inhibitors were also effective as tyrosine kinase inhibitors (*e.g.*, catechin, cromolyn sodium, etoperidone, morin, palmitoylcarnitine, phloretin, quercetin, and tamoxifen). Chemicals that belong to the amino acid category, such as *N*-acetyl-L-cysteine and DFMO, were also potent inhibitors.

The inhibition of TPA-induced ODC activity was studied in RTE 2C5 cells, because 2C5 cells are known to respond to ODC induction by TPA and this response can be inhibited by agents such as retinoic acid (12). Ornithine decarboxylase activity has been traditionally assayed by the release of $[^{14}C]CO_2$ using [L-^{14}C]ornithine as a substrate (39). However, CO_2 release can be due to the action of enzymes (*e.g.*, transaminase) other than ODC, and it makes the method vulnerable to spurious ODC levels. Therefore, an alternative procedure for determining ODC activity was adopted that measures the other product of ornithine decarboxylation, putrescine[4]. Fifty-two chemicals were identified as strong inhibitors in this assay. Retinoids, in general, exhibited strong inhibition of ODC activity (*e.g.*, *N*-(4-hydroxyphenyl)retinamide, retinol, and BASF 47851). A category of compounds showing dose-dependent inhibition were the sulfur compounds, especially the thiols and thiones (2-mercaptoethane sulfonic acid, l-2-oxothiazolidine-4-carboxylate, etc.). Among the natural products, compounds belonging to terpenes were strong inhibitors of ODC. As the ODC assay procedure adopted here specifically measures putrescine levels, it appears that this biochemical assay is a reliable screening tool for chemopreventive agents.

The inhibition of propane sultone-induced PADPR activity was studied using primary human foreskin fibroblasts. Human foreskin fibroblasts were found to be suitable for the assay, as the cells had less endogenous enzyme activity than human bronchial epithelial (BEAS-2B) cells. Near-confluent cells treated with propane sultone for 18 h showed maximum induction of enzyme activity (a 4-fold increase compared to media control). Forty-seven compounds were classified as positive compounds; these compounds were also strong inhibitors. There was no particular class of compounds that were identified by this assay. Because this enzyme has a variety of functions in the cell (*e.g.*, differentiation and DNA repair), it is possible that this assay might detect a broad range of compounds with varied biological activity. There are also indications that the positive inhibitor, 3-aminobenzamide, used in this assay can act as a cocarcinogen in UV-B-induced mouse skin carcinogenesis (40).

Compounds were tested for inhibition of B(*a*)P binding to the DNA of immortalized human BEAS-2B cells. These cells were chosen because they are known to have carcinogen-metabolizing capabilities. The 21 compounds classified as strong inhibitors include a large number of phenolic compounds followed by sulfur compounds. The results are consistent with the reports that a plant phenol, ellagic acid, was effective in the inhibition of B(*a*)P-induced carcinogenesis. Because the chemopreventive activity of ellagic acid is presumed to be due to its ability to accelerate the detoxification of B(*a*)P diol epoxide

Table 2 *List of compounds identified as positive in all assays*[a]

Compound	TK	ODC	GSH	FR[b]	PADPR	DNA-binding
Bismuthiol	+++	+++	+	+++	+++	+++
Esculetin	+	+++	+	+	++	+++
Etoperidone	+++	+++	+	++	+++	+++
Folic acid	+++	+++	++	++	+++	++
Hydrocortisone	+++	+++	+	+++	+++	+
Indole-3-carbinol	+++	+	++	+++	+++	+++
Tocopherol succinate	+	++	++	++	+++	+++
Vitamin C	+++	+++	++	+	++	+

[a] See footnotes to Table 1.

[b] FR, free radical.

[4] S. Sharma., J. D. Stutzman, and K. R. Garris. An alternative ODC assay to screen agents with potential chemopreventive activity, manuscript in preparation.

[5] J. D. Stutzman and S. Sharma. A modified tyrosine kinase assay for screening potential chemopreventive agents, manuscript in preparation.

by forming a B(*a*)P diol epoxide-ellagic acid adduct, it is possible that the phenolic compounds identified in this assay also exert their action by the same mechanism. The second category of compounds, the sulfur-containing agents, might act by inducing detoxifying enzymes for the synthesis of GSH or by catalyzing other conjugate reactions, resulting in the elimination of carcinogen products. Therefore, this assay is very specific in screening compounds that can prevent adduct formation or induce detoxifying enzymes. The assay is also useful in identifying vitamins and their analogues as potential chemopreventive agents (vitamin A: *all trans*-retinoic acid, RO16–9100; vitamin E: α- tocopherol succinate, α-tocopherol succinate PEG 1000). Their actions may be pleiotropic, and the inhibition of carcinogen-DNA binding may be an important mechanism of action.

The induction of reduced GSH in a normal rat liver cell line (BRL3A cells) was also evaluated to identify potential chemopreventive agents. When buffalo rat liver cells were treated with 25 μM Oltipraz, there was a 40% increase in the basal level of GSH (preliminary studies). Nineteen other compounds were identified as strong inducers. As expected, sulfur-containing compounds were prominent inducers of GSH (*e.g.*, *N*-acetyl-L-cysteine, diallyl sulfide and diallyl disulfide, Oltipraz, and sodium thiosulfate). Many of the phenolic compounds were also strong GSH inducers (*e.g.*, chlorogenic acid, curcumin, and vanillin). Although retinoids are known to exhibit a variety of chemopreventive activities, only temaroten was proven to be highly effective in this assay. Many of the moderate GSH-inducing compounds, rated as "++," belonged to the sulfur-containing chemical class and may be considered as strong inducers of GSH (*e.g.*, anethole trithione, l-2-oxothiazolidine-4-carboxylate, taurine, suramin, thioctic acid, and thiolutin).

The inhibition of free radicals induced by TPA was measured as a biochemical end point in either human leukemic or primary human fibroblasts. Forty-nine % of the compounds tested were identified as good oxygen radical scavengers. Because this assay uses cytochrome *c* reduction as a spectrophotometric measurement of oxygen radical formation in 96-well culture dishes, a large number of compounds can be analyzed rapidly. An additional time advantage was gained by using leukemic cells instead of skin fibroblasts (which require a minimum of 30 min preincubation for attachment), reducing the total assay time to only 30 min without affecting the quality of results. Overall, this assay can be considered as the most cost effective as well as the most rapid assay developed thus far. A significant number of phenolic and sulfur compounds were strong O_2 radical scavengers. Some antiinflammatory compounds were also identified as free radical inhibitors (*e.g.*, hydrocortisone, α-tocopherol succinate, and PEG 1000).

In conclusion, we have modified, standardized, and developed six biochemical assays for screening 90 compounds for potential chemopreventive activity. Tyrosine kinase and PADPR assays are ideal for general screening because they are useful in detecting compounds with a variety of chemical structures. The GSH, ODC, free radical, and DNA-binding assays are very specific assays, which allow the identification of compounds belonging to a certain chemical class (GSH-sulfur; free radical and DNA-binding-phenolic and sulfur) or having a certain biological activity (ODC-antipromoters and antioxidants). In general, retinoids were quite active in most of the assays. A number of compounds, as shown in Table 2, were positive in all six assays. Three of the eight were considered vitamins. Such agents are considered very promising cancer-preventing agents because of their multiple activities. These data provide highly useful information for further development and prioritization of compounds as chemoprevention agents in animal studies and later in human clinical trials.

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